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ABSTRACT

Peripheral nerve sheath tumors are the major tumors in neurofibromatosis type 1 (NF1). Malignant transformation of these tumors leads to extremely poor prognosis without therapy. The main goal of this project is to determine the efficacy of recombinant oncolytic herpes simplex type 1 viruses (HSV) for the therapy of nerve sheath tumors. To that aim we will generate reliable tumor models for malignant peripheral nerve sheath tumors (MPNST). Several existing and novel oncolytic HSV vectors will then be tested on these models for therapeutic utility.

To examine the combination of anti-angiogenic and oncolytic virus therapy, recombinant G47Δ vectors expressing anti-angiogenic factors dominant-negative fibroblast growth factor receptor (dnFGFR) and platelet factor 4 (PF4) have been generated. Expression of dnFGFR from G47Δ increases cytotoxicity *in vitro* to human endothelial cells and murine $Nf1^-$ MPNST cell lines. Inhibition of MPNST M2 tumor growth *in vivo* was significantly enhanced by the expression of dnFGFR or PF4 compared to G47Δ alone. This provides support for the clinical application of this novel combinational therapy.

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INTRODUCTION

The defining tumors in NF1 are discrete and plexiform neurofibromas and malignant peripheral nerve sheath tumors (MPNST). Much of the morbidity and mortality in NF1 patients is caused by these peripheral nerve tumors. Improvement of existing therapies and development of new therapies for these tumors is hindered by difficulties to systematically characterize tumor development and to control treatment strategies, and by the lack of appropriate animal models. The goal of the work funded by this grant is to develop appropriate animal models for the testing of experimental therapies for MPNST. Oncolytic viruses will be used to explore their efficacy in MPNST therapy in these models.

Genetic and cell biological technologies have recently made available a number of mouse models in which peripheral nerve tumors and MPNSTs are formed. In this proposal we will use these and develop other models to determine the therapeutic efficacy of oncolytic herpes simplex type I viruses (HSV) alone and in combination with anti-tumor immune- and anti-angiogenesis treatment, respectively. Oncolytic HSV vectors have been shown to be effective treatments for a number of nervous system tumors in animal models, such as glioblastoma and medulloblastoma (Mineta, Rabkin et al. 1995; Mashour, Moulding et al. 2001). These viruses have also been shown to be safe for use in humans in a phase 1 clinical trial (Markert, Medlock et al. 2000).

Taken together, we will establish whether oncolytic HSV vectors are effective against MPNST in animal models, whether anti-tumor immunity can be induced against these tumors, and whether expression of anti-angiogenic factors from oncolytic HSV vectors provides additional benefit.

BODY

1. Testing cell lines for sensitivity to oncolytic viruses and cells for tumorigenicity (Task 1).

Complete

2. Production of oncolytic virus stocks (Task 4)

As stated in the last annual report, instead of generating defective HSV vectors expressing anti-angiogenic factors, we have generated recombinant oncolytic HSV vectors, which are much easier to produce and are potentially translatable to the clinic. We have completed this Task.

3. Therapy by local delivery of oncolytic virus (Task 5).

Murine cell model (nude mice).

In the first annual report, we described studies examining the inhibition of subcutaneous MPNST M1 and M4 tumors by G47Δ. Here we demonstrate the significant inhibition of subcutaneous MPNST M2 tumors by bG47Δ-empty (Fig 3).

Murine cell model (syngeneic, immune competent)

We are currently testing the growth of the Nf1/Trp53 knockout MPNST tumor cell lines in C57Bl/6 x 129sv F1 mice (cell lines were derived from transgenic mice maintained on a mixed C57Bl/6 x 129sv background (Vogel, Klesse et al. 1999)) in order to generate an immune-competent MPNST model. If we are successful, we will be able to examine systemic delivery of oncolytic HSV (Task 6).

4. Anti-angiogenesis targeting of MPNST (Task 7).

In vitro activities of oncolytic HSV expressing dnFGFR or PF4.

We have devoted considerable effort to developing anti-angiogenesis therapy in the context of oncolytic HSV for MPNST, for many of the reasons outlined in the proposal (ie., neurofibromas and MPNSTs are highly angiogenic and neurofibromas overexpress many angiogenic factors (Kurtz and Martuza 2002)), and made substantial progress. In the last annual report, we described the generation of a set of oncolytic HSV vectors expressing anti-angiogenic factors; bG47Δ-dnFGFR (G47Δ expressing dominant-negative FGFR), bG47Δ-PF4 (G47Δ expressing platelet factor 4), and the control bG47Δ-empty (G47Δ with no transgene).

These vectors replicate similarly in mouse MPNST Nf1-/p53- cell lines (M1 and M2) and proliferating HUVECs (Fig 1). This demonstrates that expression of the transgene did not alter

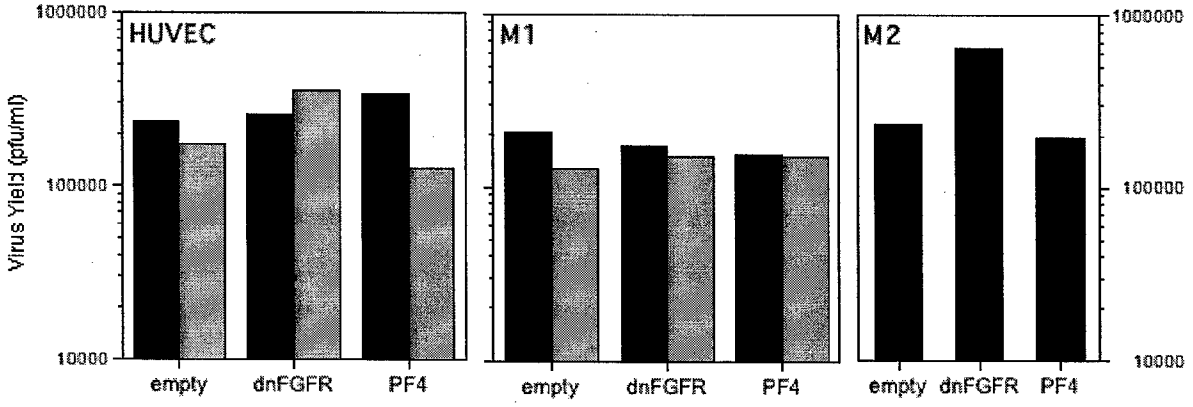

Figure 1. Virus yield on MPNST and HUVEC cells. Proliferating human endothelial cells (HUVEC grown in EGM-2 media; Cambrex, Walkersville MD), MPNST M1 and M2 cells were seeded into 12-well plates (1 x 10^5 cells/well) and at 70-80% confluency infected with bG47Δ-empty, bG47Δ-dnFGFR, and bG47Δ-PF4 at a multiplicity of infection (MOI) of 1 pfu/cell (in duplicate) for 2 hours. Virus inoculum was replaced with media, virus harvested 48 hr post-infection and titered on Vero cells. The results from 1 or 2 separate experiments are plotted.

virus replication in these cells and their oncolytic activity should be the same. Any differences in efficacy should therefore be due to the direct effect of the expressed transgene on target cells. This is illustrated in Fig. 2, where we have compared the in vitro cytotoxicity of the vectors on a number of cell lines. The effect of both dnFGFR and PF4 is greatest in HUVECs, where the EC50 was <20% that seen with bG47Δ-empty. Interestingly, the MPNST cells (M1 and M2) are quite sensitive to dnFGFR expression (Fig. 2 upper) and only minimally sensitive to PF4 (Fig. 2 lower). This suggests that in immune-deficient mice bearing M1 tumors, bG47Δ-dnFGFR should have three activities; oncolytic, anti-angiogenic, and anti-tumor.

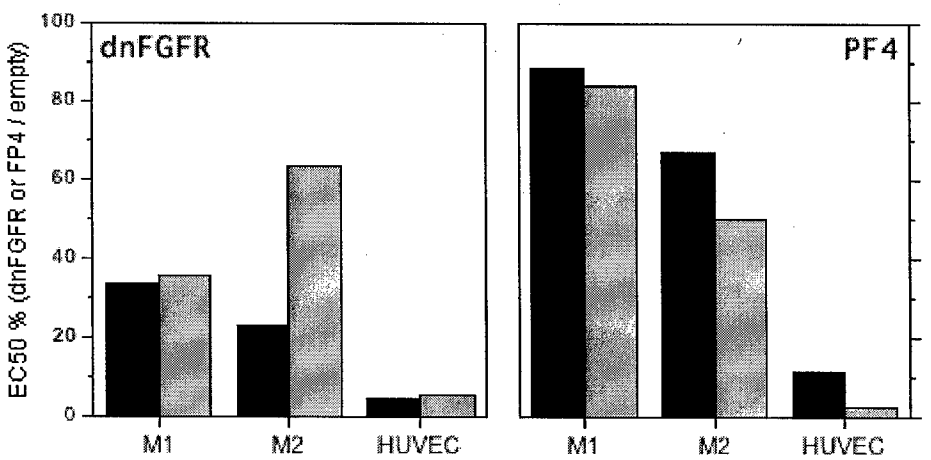

Figure 2. In vitro cytotoxicity of dnFGFR and PF4 expressing G47Δ. Cells were cells were seeded into 96-well plates at 5,000 – 7,500 cells/well. After 24 hours, they were infected with bG47Δ-empty, bG47Δ-dnFGFR, and bG47Δ-PF4 at 3-fold dilutions from MOI = 30 to 0.001 (in quadruplicate). An MTT assay was performed at 72 hours post-infection to determine cell viability and dose-response curves obtained. The EC50 for each cell line was determined and the ratio of transgene expressing / empty vector plotted. The results from 2 separate experiments are plotted.

In vivo activities of oncolytic HSV expressing dnFGFR or PF4.

We established subcutaneous M2 tumors in athymic mice, which were then treated with intratumoral injections of PBS or 10^7 pfu of bG47Δ-empty, bG47Δ-dnFRGR, or bG47Δ-PF4 (Fig 3). All three viruses significantly inhibited the growth of M2 tumors compared to mock-treated ($p < 0.01$) and bG47Δ-dnFGFR was significantly better than bG47Δ-empty. This inhibition of tumor growth was reflected in increase survival (Fig 4),with an increased median survival from 22 days for PBS to 31 days for bG47Δ-dnFGFR.

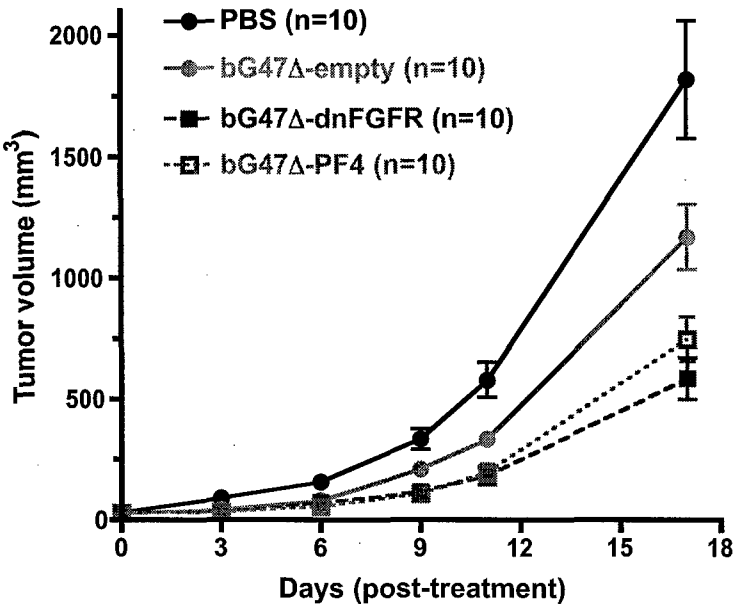

Figure 3. Inhibition of MPNST tumor growth after intratumoral injection of oncolytic HSV expressing anti-angiogenic factors. M2 cells (10^6) were implanted into flanks of 6-8 week-old nude mice. When tumor size reached 50–100 mm^3, the animals were randomized into 4 groups and treated with PBS or 10^7 pfu bG47Δ-empty, bG47Δ-dnFGFR, and bG47Δ-PF4. Four intratumoral injections were given every 3 days. All vectors were significantly better at inhibiting tumor growth compared to PBS (* $p < 0.01$; student's t test) and dnFGFR and PF4 expressing G47Δ was significantly better than the control empty vector (* $p < 0.05$).

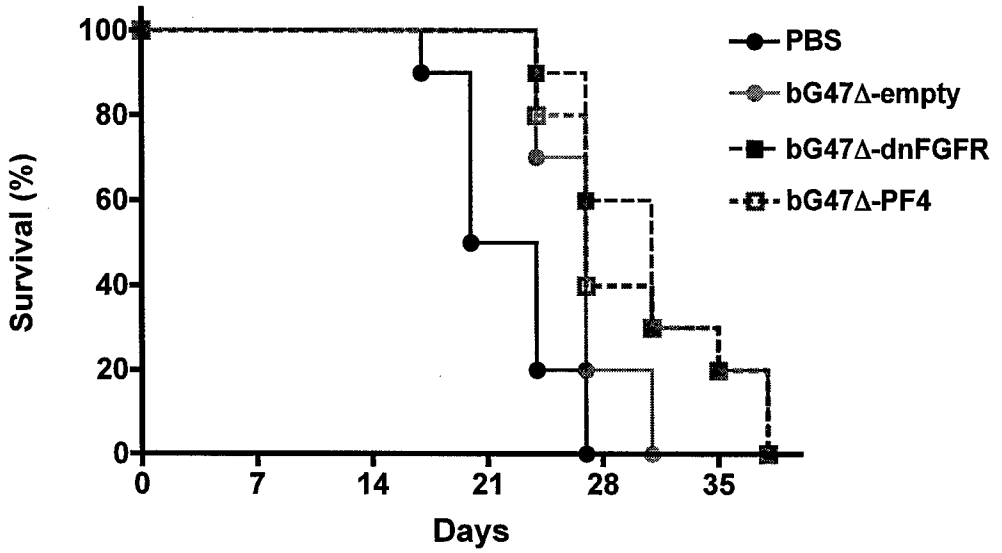

Figure 4. Kaplan-Meier survival graph of mice bearing subcutaneous MPNST M2 tumors. Mice from the experiment in Figure 3 were followed until sacrifice (tumors ≥ 2.1 cm^3). Treatment with oncolytic HSV significantly increased survival compared to PBS ($p < 0.01$; log-rank test) and bG47Δ-dnFGFR was significantly better than bG47Δ-empty ($p < 0.05$; log-rank test).

Anti-angiogenenic activity of dnFGFR and PF4 expressing G47Δ

So far we have been unable to detect sufficient numbers of immunostaining (anti-VWF or anti-CD31) vessels in the M2 tumors. To confirm that these vectors are actually inhibiting angiogenesis *in vivo*, we have used a human glioma cell line (U87), which is highly vascular (Fig 6, PBS). Even G47Δ alone inhibited angiogenesis (Fig 5, 6), likely through replication in

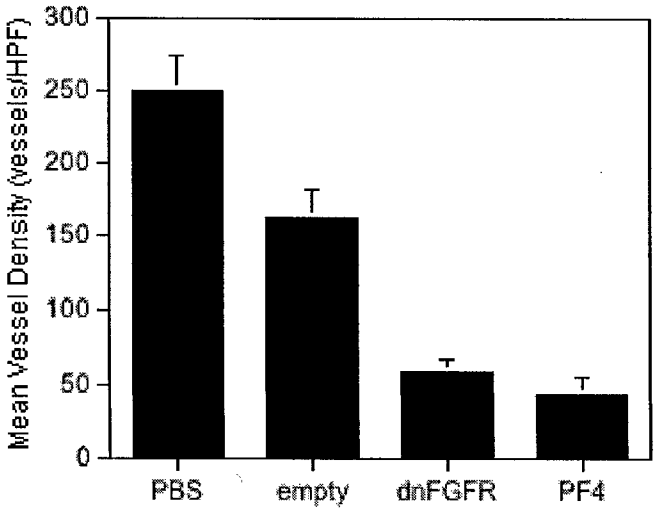

Figure 5. Microvessel density in treated human U87 glioma subcutaneous tumors. U87 cells (10^6) were implanted in 6-8 week old nude mice. When tumors reached 50-100 mm^3, the mice were randomized to 4 groups (N = 7 / group) and treated on days 0 and 3 with PBS or 5 x 10^5 pfu bG47Δ-empty, bG47Δ-dnFGFR, and bG47Δ-PF4. Tumors were harvested on day 7, snap-frozen, sectioned, and stained with anti-VWF (as described in Fig 5). Microvessel counting was performed on 200X fields (0.724 mm^2 area). The mean vessel density (MVD) was the mean value of 5 individual, non-overlapping fields in the area of vascularization. Oncolytic HSV treated tumors had significantly fewer microvessels than PBS ($p < 0.001$) and bG47Δ-dnFGFR or bG47Δ-PF4 significantly less than bG47Δ-empty ($p < 0.001$).

proliferating endothelial cells (as seen *in vitro* in Fig 1). Microvessel density after bG47Δ-dnFGFR or bG47Δ-PF4 treatment was only about 20% that seen in the mock (PBS) group and was significantly reduced compared to bG47Δ-empty (Fig 5).

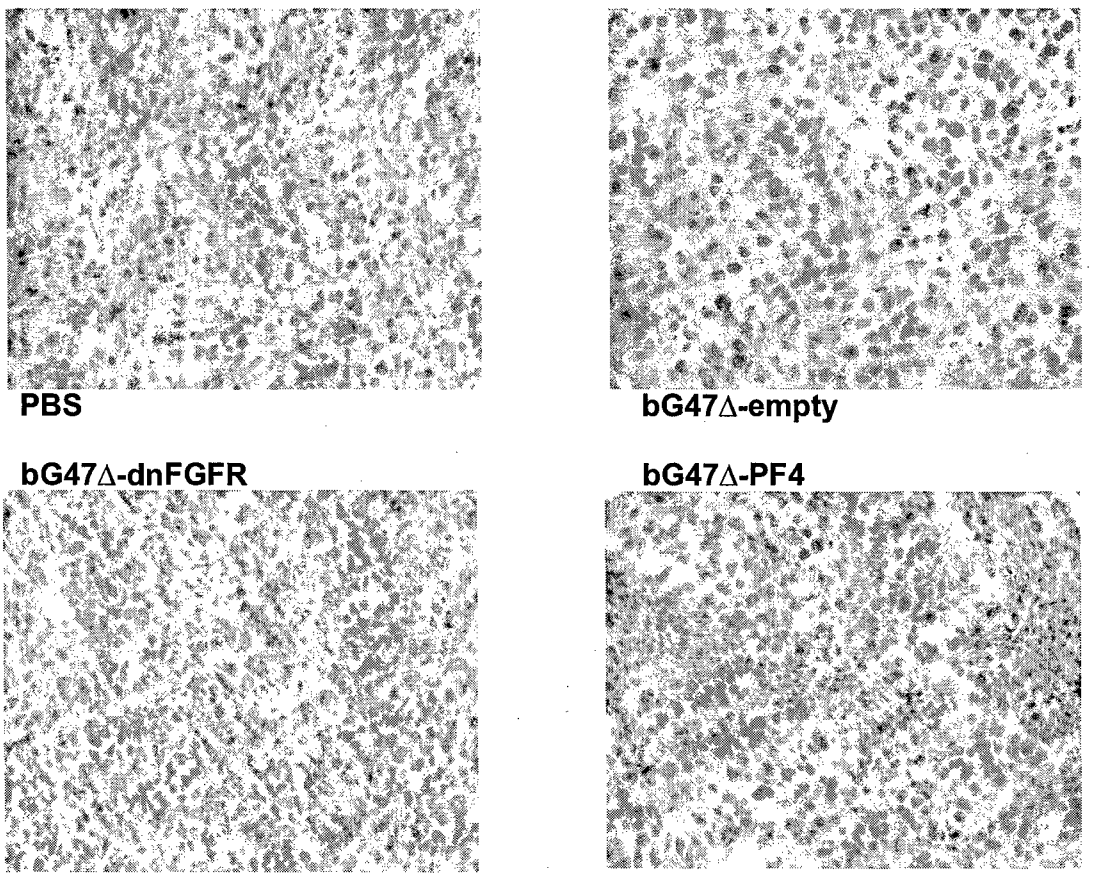

Figure 6. Tumor vasculature. U87 cells (10^6) were implanted in 6-8 week old nude mice. When tumors reached 50-100 mm^3, the mice were randomized to 4 groups (N = 7 / group) and treated on days 0 and 3 with PBS or 5 x 10^5 pfu bG47Δ-empty, bG47Δ-dnFGFR, and bG47Δ-PF4. Tumors were

harvested on day 7, snap-frozen, and sectioned. Sections were fixed in cold methanol, blocked with serum-free blocking agent (Dako, CA), quenched for endogenous hydroxide and incubated with anti-VWF (rabbit anti-mouse, 1:100; Dako, CA) overnight. They were then washed, incubated with secondary antibody (HRP conjugated goat anti-rabbit, 1:500; Amersham, MA), developed by DAB substrate-chromagen system (Dako, CA) and counterstained with hematoxylin.

KEY RESEARCH ACCOMPLISHMENTS

- The oncolytic vectors bG47Δ-empty, bG47Δ-dnFGFR, bG47Δ-PF4, generated from HSV-BAC are similarly infectious, with similar virus bursts in murine Nf1/Trp53 knockout MPNST cell lines and proliferating human HUVEC cells.
- Expression of dnFGFR and PF4 from G47Δ increases cytotoxicity to HUVEC cells by about 5-fold.
- Expression of dnFGFR from G47Δ has a much greater cytotoxic against MPNST cell lines M1 and M2 than PF4, indicating that dnFGFR should have a dual inhibitory action against these MPNST tumors.
- While bG47Δ-empty is somewhat efficacious in inhibiting MPNST M2 tumor growth, dnFGFR and PF4 significantly improve efficacy.
- The anti-angiogenic activity of bG47Δ-dnFGFR and bG47Δ-PF4 in vivo could be demonstrated in U87 glioma tumors.

REPORTABLE OUTCOMES

Liu, T-C, Zhang, T, Fukuhara, H, Kuroda, T, Martuza, RL, Kurtz, A, Rabkin, SD. Dominant-negative FGF receptor expression by oncolytic HSV vector for the treatment of malignant peripheral nerve sheath tumors. Mol Ther 11: S295, '05

CONCLUSIONS

Previously we demonstrated that dnFGFR transfection of Nf1/Trp53 knockout MPNST cells greatly slowed their growth *in vivo*. We have now constructed oncolytic HSV vectors expressing dnFGFR and PF4, another anti-angiogenic factor. Comparing the efficacy of these vectors (bG47Δ-dnFGFR and bG47Δ-PF4) to the control oncolytic vector bG47Δ-empty, we have tested our hypothesis that inhibition of FGF signaling in MPNST cells and anti-angiogenic

activity will enhance oncolytic HSV therapy. Using the murine MPNST cell line M2, we showed that expression of dnFGFR or PF4 significantly enhanced the inhibition of subcutaneous tumor growth compared to bG47Δ-empty and dnFGFR significantly extended survival of these tumor-bearing mice. This forms the basis for a novel combinational therapy for MPNSTs, oncolytic HSV and local anti-angiogenic factor expression.

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APPENDIX

Abstract for American Society of Gene Therapy Annual Meeting, 2005

[761] Dominant-Negative FGF Receptor Expression by Oncolytic HSV Vector for the Treatment of Malignant Peripheral Nerve Sheath Tumors

Ta-Chiang Liu, Tingguo Zhang, Hiroshi Fukuhara, Toshi Kuroda, Robert L. Martuza, Andreas Kurtz, Samuel D. Rabkin. Molecular Neurosurgery, Massachusetts General Hospital, HMS, Charlestown, MA; Pathology, Shandong University, Jinan, Shandong, China; Urology, Fraternity Memorial Hospital, Tokyo, Japan; Robert Koch Insititute, Berlin, Germany

Oncolytic herpes simplex viruses (HSV) have emerged as a promising platform for cancer therapy. Their large size permits insertion of therapeutic transgenes to augment the cytotoxicity of the backbone vector. Here we examine the activity of a dominant-negative FGF receptor (dnFGFR). Fibroblast growth factor (FGF) has been shown to stimulate the proliferation of Schwann cells *in vitro*, in addition to its mitogenic activity in endothelial cells, indicating its importance in malignant peripheral nerve sheath tumors (MPNST). We established MPNST cell lines transformed with dnFGFR or the control plasmid and showed that dnFGFR decreased cell proliferation and migration *in vitro* and reduced vascularity and delayed tumor growth *in vivo*. The dnFGFR transgene was inserted into oncolytic HSV G47 (deleted in 47/Us11 promotor, 34.5, and a LacZ insertion inactivating ICP6), using the G47-BAC (bacterial artificial chromosome) system. G47-dnFGFR showed enhanced killing of both tumor and proliferating endothelial cells *in vitro* compared to the G47-empty control vector. *In vivo*, G47-dnFGFR was more efficacious than its non-expressing parent G47-empty at inhibiting MPNST growth. Therefore oncolytic HSV encoding antiangiogenic dominant-negative FGFR transgene is a new strategy for tumor treatment.

Keywords: HSV Vectors; Cancer Gene Therapy; Targeted Gene Expression